Effect of unesterified cholesterol on the activity of cholesteryl ester transfer protein

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Cholesteryl ester transfer protein (CETP) catalyses the transfer of cholesteryl ester from high-density lipoprotein to triacylglycerol-rich lipoproteins and the transfer of triacylglycerols in the reverse direction. The activity of CETP has been studied using a continuous fluorescence assay which measures the excimer fluorescence of cholesteryl 1-pyrene decanoate in a synthetic donor microemulsion as the indicator of cholesteryl ester transfer. Emulsions were composed of cholesteryl oleate and egg phosphatidylcholine and had an average particle size of 14 ± 1 nm as calculated from the molar volume of the components. The effect of changing the physical state of the emulsion surface was examined by including unesterified cholesterol in the donor and acceptor particles. The rate of CETP-induced transfer of the fluorescent cholesteryl ester between microemulsion particles increased when unesterified cholesterol was present at concen-

trations up to 17 mol % relative to phospholipid. The presence of cholesterol also changed the exchange kinetics from an apparent single-exponential to a double-exponential phenomenon. Binding of CETP to the emulsion surface was accompanied by an enhancement of fluorescence which was used to measure the binding equilibria. The enhancement of exchange due to the presence of cholesterol did not correlate with any increased binding of CETP to the emulsion surface. The presence of unesterified cholesterol in the donor did not affect the rate of transfer of the fluorescent cholesteryl ester when unlabelled emulsion was replaced by high-density lipoprotein as the acceptor. The studies demonstrate the use of microemulsions of defined size and composition for the study of the mechanism of action of CETP.

INTRODUCTION

Cholesteryl ester transfer protein (CETP) is present in the blood plasma of certain mammalian species and facilitates the transfer and exchange of cholesteryl esters (CEs) and triacylglycerols between different lipoprotein classes [1]. CEs are mainly formed on the surface of high-density lipoprotein (HDL) particles in plasma by the action of lecithin:cholesterol acyltransferase (LCAT) and are transferred to triacylglycerol-rich lipoproteins by the action of CETP which also promotes the net transfer of triacylglycerols in the reverse direction [1,2]. The triacylglycerol-rich lipoproteins are simultaneously transformed by lipolytic enzymes to chylomicron remnants and intermediate-density lipoprotein (IDL) and are removed by the hepatic receptors either directly, or in the case of IDL, after conversion into low-density lipoprotein (LDL). CETP therefore plays a central role in the vectorial transfer of CEs from the plasma and peripheral tissues back to the liver for catabolism [3]. The proportion of CE to triacylglycerol in the lipoprotein substrates appears to determine which neutral lipid is carried by CETP [4,5].

The mechanism by which CETP interacts with a donor particle and transfers the neutral lipid to a suitable acceptor is unknown. Both shuttle and ternary complex (donor–CETP–acceptor) mechanisms have been proposed [6,7]. Regardless of the mechanism, an important issue is whether CETP penetrates the surface phospholipid monolayer of a lipoprotein to access CE or triacylglycerol in the core of the emulsion, or binds to the particle surface and acquires the small amounts of these compounds

which are soluble in the phospholipid monolayer. In either case, the integrity or physical state of the emulsion surface is likely to influence the acquisition of substrates by CETP. Given the well-documented effects of cholesterol on the physical state of lipid bilayers, and the primary location of cholesterol in the surface monolayer of lipoproteins, it might be expected that cholesterol will influence the activity of CETP by affecting its interaction with the emulsion surface. In this paper we modify the donor/acceptor surface by changing the content of unesterified cholesterol (UC) and examine its effect on the transfer of CE between emulsion particles.

The effects of UC on CETP activity in natural lipoprotein systems is complex due to varying ratios of CE to triacylglycerol present in donor and acceptor particles and to possible modulation of CETP activity by apolipoproteins [8,9]. Existing reports of the effect of cholesterol on CETP activity are illustrative of the complexity of the experimental systems used. Morton modified the cholesterol contents of very-low-density lipoprotein (VLDL), LDL and HDL by incubation with cholesterol-containing liposomes [10]. An increase in the cholesterol content of donor LDL or VLDL caused a concentration-dependent inhibition of CE transfer from these particles [10]. On the other hand, an increase in the cholesterol content of donor HDL had little effect on CE transfer. We have therefore examined the CETP-induced transfer of CE between synthetic microemulsions of well-defined size and composition where the effects of UC can be more clearly established. The classical assay of CETP activity involves the transfer of a radiolabelled CE between donor and acceptor

Abbreviations used: CETP, cholesteryl ester transfer protein; EYPC, egg-yolk phosphatidylcholine; UC, unesterified cholesterol; CO, cholesteryl oleate; CE, cholesteryl ester; Py-CE, cholesteryl 1-pyrene decanoate; VLDL, very-low-density lipoproteins; IDL, intermediate-density lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoproteins; HDL₃, high-density lipoprotein, subfraction 3; LCAT, lecithin:cholesterol acyltransferase.

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particles, the physical separation of donors and acceptors, and the determination of the radiolabel in either population [11]. The method lacks the time resolution required for kinetic analysis and is limited to donors and acceptors that can be separated by a precipitation or centrifugation method. Ohnishi and colleagues reported an *in situ* method for determining CETP activity involving measurement of the monomer:excimer ratio of emission of a pyrenyl analogue of CE [8,12]. We report a modification of this method which uses the excimer intensity as an indicator of transfer efficiency under conditions where the excimer fluorescence arises only from the donor particles.

EXPERIMENTAL

Materials

Cholesteryl 1-pyrene decanoate (Py-CE) was purchased from Molecular Probes, Oregon. Egg-yolk phosphatidylcholine (EYPC), cholesteryl oleate (CO), UC and the sodium salt of heparin (175 units/mg) were from Sigma, St. Louis, MO, U.S.A. [1,2-³H]Cholesterol (40 Ci/mmol) was from Amersham, Australia, and was used without further purification.

Lipoproteins

Blood was obtained from healthy male subjects who had been fasting for 15 h overnight. Plasma was separated by centrifugation at 4 °C, and adjusted to appropriate densities by adding solid KB4 [13]. Lipoproteins were isolated by sequential ultracentrifugation [14] using a Beckman Ti 70.1 rotor at a speed of 48 000 rev./min. LDL ($d = 1.019\text{--}1.055$) was isolated with a single spin of 24 h at the lower density and two successive spins for the same period at the higher density. HDL subfraction 3 (HDL₃) ($d = 1.13\text{--}1.21$) was isolated with a single 24 h spin at the lower density and two 40 h spins at the higher density.

Radiolabelled HDL₃ was prepared by the method of Albers et al. [15]. Briefly, [³H]cholesterol was dissolved in ethanol and added to a preparation of a freshly isolated and dialysed plasma fraction of $d > 1.13$. The mixture was then incubated at 37 °C to allow cholesterol esterification catalysed by LCAT. The HDL₃ fraction was re-isolated by centrifugation and found to contain labelled CEs which accounted for more than 95% of the radioactivity applied. Lipoprotein fractions were dialysed against 10 mM Tris/HCl buffer (pH 7.4) containing 0.15 M NaCl, 1.0 mM EDTA and 3.0 mM NaN₃ (buffer A).

Preparation of [³H]CE

Aliquots of [³H]cholesterol and freshly isolated plasma fraction of $d > 1.13$ were mixed and incubated as described above to allow cholesterol esterification by LCAT. The mixture was then extracted with a solution of isopropyl alcohol/n-heptane/0.5 M sulphuric acid (40:10:1, by vol). [³H]CEs were separated by t.l.c. using hexane/diethyl ether/methanol/acetic acid (67:30:2:1, by vol.) as the solvent [16].

Microemulsions

Model LDL-type emulsions were prepared essentially according to the method of Ginsburg et al. [17]. Equimolar amounts of EYPC and CO dissolved in CHCl₃/methanol (1:1, v/v) were placed in glass vials, the solvent removed by evaporation under a stream of nitrogen, and the material vacuum desiccated at room temperature for 24 h. For preparing microemulsions containing labelled CE, the initial lipid mixture also contained Py-CE (3.5–20% by weight of total CO) and/or [³H]CE (2.85 μ Ci,

13% by weight of total CO). In the case of lipid emulsions containing UC the initial lipid mixture contained 2.5, 5 or 7.4 mol% of UC relative to EYPC. The lipids were dispersed in buffer B (10 mM Tris/HCl, pH 8.0) containing 0.1 M KCl and 3.0 mM NaN₃ to a total lipid concentration of 0.1%. The suspension was sonicated using an MSE Soniprep at maximum power for 120 min under nitrogen. The temperature was maintained at 51–60 °C with a circulating water bath. The sonicated lipid suspension was centrifuged for 10 min in a bench centrifuge (3000 rev./min) to remove any titanium fragments derived from the sonication probe and then spun in an SW 60 rotor of a Beckman ultracentrifuge at 43 000 rev./min for 30 min. The upper 10% of the volume containing the floating lipid was discarded and the density of the remaining solution adjusted to 1.21 g/ml with solid KBr [13]. The samples were spun in a Beckman TL-100 table-top ultracentrifuge using a TLA-100.2 rotor for 2 h at 100 000 rev./min. The $d < 1.21$ fraction was recovered by tube slicing and applied to a Superose 6 HR 10/30 column connected to an f.p.l.c. system (Pharmacia-LKB, Uppsala, Sweden). Fractions were analysed for phospholipid, CE and free cholesterol. Those fractions showing a relatively constant ratio of CE/phospholipid were pooled and concentrated by centrifuging in a TL-100 rotor as described above. On the basis of the lipid composition and the molar volume of the components, the particle size was calculated to be 14.1 ± 1.0 nm.

Microemulsions were stored in buffer A and used within 4 days of preparation to avoid the aggregation of the particles known to occur during longer periods of storage [18]. The sonication procedure did not result in degradation of the lipids as determined by t.l.c. using solvent systems of chloroform/methanol/water/acetic acid (65:25:4:1, by vol.) for polar lipids and hexane/diethyl ether/methanol/acetic acid (67:30:2:1, by vol.) for non-polar lipids. The sonication procedure did not result in oxidation of lipids as detected using the absorption method of Klein [19].

Preparation of CETP

CETP was partially purified from the cryosupernatant of human plasma as described previously [20] to produce a preparation that revealed four bands on SDS/PAGE. The major band had a relative molecular mass of 72 000 characteristic of CETP. The final preparation was stored at –80 °C and had no detectable LCAT activity.

Fluorescence measurements

Fluorescence measurements were made with a Perkin–Elmer LS-5 spectrofluorimeter equipped with a thermostatically controlled cell holder. Emission spectra were recorded between 360 nm and 540 nm with an excitation wavelength of 340 nm at a scanning speed of 60 nm/min. All measurements were corrected for the scatter of excitation light. Measurements of the excimer emission were made at excitation and emission wavelengths of 340 and 470 nm respectively.

Fluorescence assay for CE transfer

A volume of Py-CE-labelled microemulsion (as donor) and either unlabelled emulsion or HDL (as acceptor) were mixed with CETP in a microcuvette and incubated at 37 °C. The transfer of Py-CE from donor to acceptor was measured as the decrease in the excimer fluorescence at 470 nm. A control incubation containing exactly the same amounts of donor and acceptor particles but without CETP was carried out to correct

for spontaneous transfer. The decrease in the excimer fluorescence in the absence of CETP was always less than 2%. The ratio of donor to acceptor was 1:8 based on the concentration of CE. Under these conditions the excimer fluorescence arises only from the donor particles and is directly proportional to the microscopic concentration of Py-CE in the donor emulsion. The control data set obtained in the absence of CETP was subtracted from each data set obtained in the presence of CETP. The resulting curves were fitted to a one- or two-exponential function using a non-linear least-squares fitting procedure.

$$F(t) = \sum a_i \exp(-t/\phi_i) + c \quad (1)$$

where $F(t)$ is the excimer fluorescence at time t , ϕ_i is the time constant of the i th component of amplitude a_i , and c is the final equilibrium excimer level.

Radiochemical assay of CE transfer

The CE transfer activity during CETP purification was measured as the capacity of a sample to facilitate the transfer of [3 H]CE from HDL₃ to LDL in a 3 h incubation at 37 °C [15]. For measuring CE transfer between microemulsions and HDL₃ the following procedure was used. Lipid microemulsions, doubly labelled with [3 H]CE and Py-CE were mixed with HDL₃ and incubated at 37 °C for up to 80 min. Donor and acceptor particles were separated by a polyanion-precipitation method involving the use of heparin and MnCl₂ (final concentrations; 0.8% heparin, 0.1 M MnCl₂) [21]. Control experiments showed that this treatment quantitatively precipitated the emulsion. The precipitate was removed by centrifugation. One aliquot of the supernatant containing HDL was measured for radioactivity and the other for fluorescence (excitation and emission wavelengths: 340 nm and 395 nm respectively).

Determination of binding affinity

The binding of CETP to emulsion surfaces was measured from the enhancement of the intrinsic fluorescence of CETP on binding to emulsion particles. Fluorescence measurements were made at 37 °C (excitation 280 nm and emission 335 nm). The procedure was similar to that described by McLean and Jackson for measuring the binding of lipoprotein lipase to bilayer surfaces [22]. Aliquots (10–20 μ l) of CETP (0.1–0.2 mg/ml) in buffer A were added to 3.0 ml of the lipid emulsion (14–30 μ M in phospholipid). The difference in the fluorescence emission of CETP in the presence and absence of lipid (ΔF) was determined from two separate titrations. The binding of protein to equivalent and independent sites on an acceptor may be written [23]:

$$K_d = \frac{[P][L_n]}{[PL_n]} = \frac{(P_T - x)(L_T/n - x)}{x} \quad (3)$$

where x is the concentration of bound protein, n is the number of lipid molecules per protein molecule in the complex, K_d is the dissociation constant, and P_T and L_T are the concentrations of total protein and phospholipid respectively. Eqn. 3 may be rearranged as follows:

$$P_T = x[K_d + (L_T/n) - x]/[(L_T/n) - x] \quad (4)$$

Values for x were obtained from the relationship, $\Delta F = Cx$, where C is the molar fluorescence intensity difference constant determined at high lipid:protein ratios where the fluorescence

difference is proportional to protein concentration ($\Delta F = CP_T$). The maximum values of ΔF in a titration with CETP were found under conditions of high lipid concentration where $\Delta F = Cx = C(L_T/n)$. Values of K_d and n were determined by non-linear least-squares fitting according to eqn. 4. Based on SDS/PAGE analysis of the protein adsorbed to microemulsions, the minor impurities in the CETP preparation did not bind significantly to the lipid and therefore did not contribute to the observed fluorescence enhancement.

Other analyses

Concentrations of phospholipids and of total and unesterified cholesterol were measured with enzymic kits (Boehringer-Mannheim GmbH, Germany) used in conjunction with a Cobas-Bio centrifugal analyser (Roche Diagnostics, Zurich, Switzerland). The concentration of esterified cholesterol was calculated as the difference between the concentrations of total and unesterified cholesterol. Apolipoprotein A1 was determined by immunoturbidometric assay [24] and protein concentration by the method of Lowry et al. [25]. The concentration of Py-CE was determined spectrophotometrically using the absorption coefficient of 38000 M⁻¹·cm⁻¹ at 342 nm [26].

RESULTS

Emission spectrum of Py-CE-containing emulsions

The emission spectrum of EYPC-CO microemulsions spiked with Py-CE (9 mol % relative to CO) is shown in Figure 1. Bands characteristic of the pyrene monomer fluorescence occur at 378 and 396 nm. The significant excimer band at 470 nm increased relative to the monomer bands when the Py-CE:CO ratio was increased (results not shown). When a Py-CE labelled microemulsion was incubated with HDL₃ and CETP at 37 °C for 60 min, the excimer emission of pyrene decreased while the monomer emission increased, consistent with the transfer of Py-CE from the emulsion to HDL₃ (Figure 1).

Figure 1 Time-dependent change of the pyrene fluorescence emission spectrum due to CETP-mediated transfer of Py-CE from donor emulsion to acceptor HDL

Py-CE-labelled microemulsion (44 nmol of CE), unlabelled HDL₃ (318 nmol of CE) and CETP (23 μ g/ml) were mixed in a final volume of 600 μ l and incubated at 37 °C. Emission spectra were recorded at 0 (—) and 60 min (---) following incubation. Excitation was at 340 nm.

Figure 2 Excimer fluorescence as a function of the concentration of Py-CE in the emulsion

The excimer fluorescence intensity of the microemulsions containing various amounts of Py-CE (relative to total CE) was measured at 470 nm. The curve is the fit of the data points to a single-exponential function. The inset is the semi-logarithmic plot of the data.

Relationship between excimer fluorescence and Py-CE content in the microemulsion

This relationship was examined over a range of Py-CE concentrations from 0 to 14 mol % with respect to the total CE in the emulsion. An exponential relationship exists between the excimer fluorescence and the mol % Py-CE in the emulsion as shown in Figure 2. The form of this relationship is determined by the bimolecular nature of the excimer formation and also by the interplay between the rate constants for excimer formation and excimer emission. The absence of a discontinuity in Figure 2 suggests that Py-CE does not phase separate within the emulsion at a particular Py-CE concentration. Moreover, the excimer intensity at zero time did not change on the addition of CETP, indicating that CETP itself did not induce phase separation of the probe.

Transfer of Py-CE from donor to acceptor

Donor and acceptor particles were physically separated to prove that CETP accomplished the transfer of Py-CE from one to the other. Aliquots of Py-CE-labelled emulsion and HDL₃ were incubated with CETP for 60 min. Following incubation the donor and the acceptor particles were separated by size-exclusion chromatography on a Superose 6 column. The eluate was monitored for CE, apolipoprotein A1 and for pyrene fluorescence. The profiles obtained before and after the addition of CETP are presented in Figure 3. CETP causes a significant proportion of Py-CE to transfer from the microemulsion to HDL, as indicated by the decrease in the pyrene fluorescence of the emulsion peak (Figure 3; fractions 7–13) and an increase in the pyrene fluorescence of the HDL peak (Figure 3; fractions 16–18). All of the apolipoprotein A1 and most of the total CE is associated with the HDL peak, which is present in excess compared with the donor. These results also show that the incubation did not produce an irreversible fusion or aggregation of donor and acceptor particles.

Dependence of transfer on CETP concentration

Mixtures of Py-CE-labelled microemulsion and HDL were incubated with various concentrations of CETP at 37 °C, and the

Figure 3 Changes in the distribution of Py-CE between microemulsion and HDL during incubation with CETP

Labelled emulsion (96 nmol of CE) and unlabelled HDL₃ (672 nmol of CE) were incubated at 37 °C for 60 min in the presence (b) or absence (a) of CETP (23 μg/ml). The final volume was adjusted to 600 μl by the addition of buffer A. Incubations were stopped by placing samples on ice. Mixtures were then subjected to size-exclusion chromatography on a Superose 6 column. The lipoproteins were eluted with buffer A at a flow rate of 18 ml/h and fractions of 0.9 ml were collected for measuring excimer fluorescence (●), CE (○) and apolipoprotein A1 (Apo A1) (△).

Figure 4 Effect of CETP concentration on the transfer of Py-CE from a microemulsion to HDL

Aliquots of labelled emulsion, unlabelled HDL₃ and various amounts of CETP (○, 14 μg/ml; △, 28 μg/ml; □, 56 μg/ml) were mixed in a microcuvette at 37 °C in proportions similar to those described in the caption to Figure 1. The excimer intensity of Py-CE at 470 nm was measured at 1 min intervals for 60 min. The symbols represent the experimental points; the solid line is the single-exponential curve fitted according to eqn. 1. The inset shows the relationship between the rate constant and the concentrations of CETP in the incubation; results are the mean of three determinations.

transfer of Py-CE was monitored continuously for 60 min by measuring the decrease in excimer intensity. In the absence of CETP, the excimer decrease was less than 1 % after 60 min

Figure 5 The effect of UC on the CETP-mediated transfer of Py-CE (a) between donor and acceptor microemulsions and (b) from a microemulsion to HDL

(a) Aliquots of Py-CE labelled microemulsion (10 nmol of CE) and unlabelled emulsion (77 nmol of CE), both containing the same amounts of UC relative to phospholipid were mixed with CETP (13 $\mu\text{g}/\text{ml}$, final volume 600 μl) and changes to the excimer fluorescence were monitored continuously for 60 min. Key to symbols: $\circ$, 0 mol% UC; $\triangle$, 6 mol%; $\square$, 12 mol%; $\diamond$, 17 mol%. The solid lines are the double-exponential fits to eqn. 1: the fitted parameters are summarized in Table 1. (b) Mixtures of labelled microemulsion (10 nmol of CE) and unlabelled HDL (82 nmol of CE) were incubated at 37 $^{\circ}\text{C}$ for 2 h to allow equilibration of UC between donors and acceptors. CETP (21 $\mu\text{g}/\text{ml}$, final volume, 600 μl) was then added and the excimer fluorescence followed for 60 min. The concentration of UC in the HDL preparation was 14.4 mol% relative to phospholipid. The figures 0 and 14 refer to the mol% of UC in the emulsions prior to incubation with HDL. The 'control' was obtained by incubation of donor and acceptor particles in the absence of CETP. The solid lines are the single-exponential fits to eqn. 1.

(Figure 4). In the presence of CETP, the decrease in excimer fluorescence was enhanced in a concentration-dependent fashion. Each data set was corrected for the small passive transfer. A single exponential function (eqn. 1) was sufficient to adequately fit the data (Figure 4). The apparent correlation times were 42.5 ± 1.2 , 36.2 ± 1.1 and 34.2 ± 1.1 min for CETP concentrations of 14, 28 and 56 $\mu\text{g}/\text{ml}$ respectively (rate constants: 0.024, 0.028 and 0.030 min^{-1} respectively). Of particular note is that the rate constant increases with CETP concentration but eventually reaches a saturating level as shown in the inset in Figure 4.

The ability of CETP to promote the transfer of other pyrenyl-labelled neutral lipids was also tested. Excimer fluorescence was also observed for a CE which had the pyrenyl moiety as an integral part of the C-17 side arm [1-pyrenemethyl 3 β -(*cis*-9-octadecanoyloxy)-22,23-bisnor-5-cholelate]. CETP did not promote the transfer of this fluorescent CE, nor did CETP transfer a pyrene-labelled triacylglycerol [1(3)-pyrenylbutanoyl-2,3(1,2)-dipalmitoyl-*sn*-glycerol]. The latter compound has proved a useful fluorescent substrate for lipolytic enzymes [27].

Table 1 The effect of cholesterol on the kinetic parameters for the CETP-mediated transfer of Py-CE between microemulsion particles

Kinetic parameters were derived by fitting experimental data (Figure 5a) to eqn. 1: $F(t) = a_1 \exp(-t/\phi_1) + a_2 \exp(-t/\phi_2) + c$. The correlation times (ϕ) are in min. Other details are described in the caption of Figure 5(a).

Parameter	Cholesterol relative to EYPC (mol%)			
	0	6	12	17
ϕ_1	—	5.0	3.2	3.2
ϕ_2	47	236	183	100
a_1	—	0.036	0.105	0.064
a_2	0.144	0.409	0.510	0.542
c	0.852	0.555	0.420	0.422

Effect of UC on Py-CE transfer

The use of microemulsions to examine the mechanism of CETP-mediated lipid transfer has the major advantage that the composition of the donor and acceptor particles can be controlled. This is of particular advantage when examining the effects of cholesterol, when both donors and acceptors must have the same cholesterol content so that a net mass transfer of cholesterol during the CETP-catalysed exchange of CE can be prevented [28]. Preliminary experiments showed that CETP could also transfer Py-CE between emulsions of the same composition, except for the presence of Py-CE in the donor. The transfer of Py-CE was examined using preparations of CO/EYPC emulsions containing various amounts of UC relative to phospholipid but having the same cholesterol content in donors and acceptors. The effect of cholesterol on the transfer of Py-CE from labelled to unlabelled emulsion was followed by the decrease in excimer fluorescence: the results are shown in Figure 5(a). In the absence of cholesterol, the decrease in excimer fluorescence followed apparent first-order kinetics, as already depicted in Figure 4. The presence of cholesterol increased the complexity of the kinetics as well as the initial rate of transfer of Py-CE from donor to acceptor. In the presence of cholesterol the best fit of the data was obtained using a double-exponential function (eqn. 1), the two time constants being in the ranges 3–5 min and 100–240 min. The fitted parameters are summarized in Table 1. The experiment was repeated three times using separate microemulsion preparations with similar results.

When HDL is used as the acceptor, the question of the effect of spontaneous exchange of cholesterol on the transfer kinetics must be considered. Studies in our laboratory (Q.-T. Li and W. H. Sawyer, unpublished work) and elsewhere [28] have shown that UC exchanges spontaneously between lipoproteins or between emulsions and lipoproteins with half-times in the range 3–45 min. Unlabelled HDL (14.4 mol% UC) was preincubated with Py-CE-labelled microemulsions (containing either 0 or 14 mol% UC) for 2 h to allow the equilibration of UC between donor and acceptor particles. In these experiments the HDL acceptor was present in an 8-fold excess (on the basis of CE content) so that at equilibrium there was little difference between the UC contents of the donor and acceptor particles (12.8 and 14.4 mol% compared with the original 0 and 14 mol% levels respectively). After the 2 h preincubation, CETP was added and the extent of Py-CE transfer was monitored by measuring the excimer fluorescence. The results in Figure 5(b) show that rate of transfer of fluorescent CE from the two donor emulsions did not

Figure 6 Comparison of the transfer of ^3H - and pyrenyl-labelled CE from emulsion to HDL

A doubly labelled donor microemulsion containing both [^3H]CE and Py-CE (8 nmol of CE), was mixed with unlabelled acceptor HDL₃ (45 nmol of CE) and incubated at 37 °C for 60 min to allow equilibration of UC levels between the donor and the acceptor. CETP (23 $\mu\text{g}/\text{ml}$) was then added and the incubations continued. The final incubation volume was 175 μl . At the time intervals indicated, the transfer was stopped by placing the samples on ice, and the mixtures were then treated with heparin-MnCl₂ to precipitate the emulsion. An aliquot of the supernatant containing HDL was assayed for radioactivity (a) and for fluorescence emission at 395 nm (b). Results are expressed as the relative amounts of label present in the donor microemulsion and are the mean $\pm$ S.D. of triplicate samples. Open and closed circles represent transfer in the absence and presence respectively of 15 mol% UC (relative to total phospholipid) in the emulsion.

differ significantly, as expected on the basis of their similar levels of UC.

Comparison of the transfer of radiolabelled and pyrene-labelled CE

The relative efficiency of transfer of Py-CE was compared with that of the transfer of [^3H]CE (Figure 6) to determine whether, at similar levels of UC, the transfer of these two labelled CEs was similar. An 8-fold excess of HDL acceptor over donor was again used together with a 2 h incubation period to equilibrate cholesterol levels. After incubation, the donor and the acceptor particles were separated by the heparin-MnCl₂ precipitation technique as described in the Experimental section. The supernatant (HDL) fraction was assayed for both radioactivity and pyrene (monomer) fluorescence. After 80 min, 23 % of the Py-CE and 33 % of the [^3H]CE had transferred to the HDL (Figure 6).

Binding of CETP to emulsion particles

The effects of cholesterol on the activity of CETP could be due to several events: (i) changes in the binding of CETP to the emulsion surface, (ii) changes in the access of CETP to CE in the emulsion particle, and (iii) changes in the rate of dissociation of CETP from the donor and/or acceptor. We direct attention to

Figure 7 The effect of UC on the binding of CETP to EYPC/CO microemulsions

CETP was added to a sample of buffer or microemulsion in separate experiments and the difference in the tryptophan fluorescence intensity used to monitor the binding as described in the text. The concentration of cholesterol (14 mol%) is relative to the phospholipid in the emulsions. The approximate purity of the CETP preparation as judged by SDS/PAGE was 80%. The molar concentration of CETP was calculated taking the relative molecular mass of the protein as 72 000 and assuming the purity of the preparation to be 80%. Open and closed circles represent the binding in the absence and presence of UC respectively.

the effect of cholesterol on the binding of CETP to the emulsion surface as measured by the enhancement of the intrinsic fluorescence of CETP. The results are presented in Figure 7. The presence of 14 mol% of cholesterol in the emulsion causes a significant reduction in CETP binding. This effect is due primarily to a decrease in the binding affinity (K_d increases from 0.93 to 18.92 μM) which more than counteracts an increase in the binding capacity (from one CETP molecule/292 phospholipids to one CETP molecule/98 phospholipids). Cholesterol also reduced the binding of CETP to triolein/EYPC microemulsions, but less so than for CO/EYPC microemulsions (results not shown).

DISCUSSION

The surface environment of lipoproteins comprises a complex and poorly defined arrangement of phospholipids and proteins together with small amounts of CEs, triacylglycerols and fatty acids. Individual determinants of CETP binding on the surface of complex lipoproteins are therefore difficult to identify. The use of model microemulsions allows examination of the effects of surface parameters such as charge and microviscosity in systems of well-defined size and composition. The ability to control the composition of the donor and acceptor particles also permits the application of a sensitive fluorescent assay which allows a continuous recording of the CETP-mediated CE transfer without the physical separation of donor and acceptor particles. In the present investigation we have used CO/Py-CE/EYPC microemulsions as donors so that the kinetics of the CE transfer to a microemulsion of the same composition or to plasma lipoproteins can be studied. The physical properties of similar microemulsions prepared from CO and dimyristoyl phosphatidylcholine (DMPC) have been reported elsewhere [17,29,30].

The excimer fluorescence of Py-CE is a sensitive measure of its microscopic concentration in the donor particle. Thus CETP-induced changes in the excimer emission reflect transfer of the lipid to the acceptor particle. The method is sensitive, reproducible and allows detailed analysis of the kinetics of the transfer reaction. A fluorescence assay for CE transfer, which makes use of the monomer/excimer ratio, has been reported previously for

emulsions that contain triacylglycerol rather than CE as the major core component [8,12]. However, allowance has to be made for the monomer fluorescence arising from the acceptor as well as the donor. The present assay uses the excimer intensity as an indicator of transfer efficiency under conditions where the excimer fluorescence arises only from the donor particles. Probe which is transferred to the large pool of acceptor becomes too dilute to give rise to excimer emission. In the case of Py-CE transfer from a microemulsion to HDL, separation of donor and acceptor by gel-permeation chromatography showed that, at least in this case, the donor and acceptor particles do not fuse or form long-lived complexes.

The position at which the pyrenyl fluorophore is incorporated into CE is important: no transfer was observed when the pyrene label was part of the C-17 side arm of the molecule, suggesting that this region may direct the specificity of the binding to CETP. The comparison of the transfer of Py-CE with that of [^3H]CE (Figure 6) indicates that the former is transferred 30% less efficiently possibly because the bulky pyrenyl group interferes with its binding to CETP. Recently, Bisgaier et al. [31] who studied the CETP-mediated transfer of cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3 α ,4 α -diaz-3-indacenedodecanoate between emulsion particles reported that the [^3H]CE is transferred more effectively compared with the fluorescent CE. These comparisons should be interpreted with care. It is likely that the unlabelled CE (CO) and the two labelled forms of CE in the donor particle (Py-CE and [^3H]CE) compete for binding sites on CETP. Thus any transfer of one in preference to the other depends on their relative affinity for CETP and on their relative microscopic concentrations in the donor. Although the presence of the fluorophore on the probe molecule may lead to a decrease in the transfer efficiency, it should also be noted that CETP shows differential specificity towards naturally occurring CEs which possess acyl chains of differing length and degree of unsaturation [32]. CETP did not mediate the transfer of a pyrene-labelled triacylglycerol [1(3)-pyrenebutanoyl-2,3(1,2)-dipalmitoyl-*sn*-glycerol]. Thus a similar assay for measuring the triacylglycerol transfer has yet to be devised.

We have preferred to obtain apparent first-order rate constants from analysis of the raw data since there is uncertainty regarding the equivalence of the excimer quantum yield in donor and acceptor particles. Ohnishi and colleagues have also observed first-order kinetics using an assay based on the measurement of the monomer/excimer ratio for Py-CE fluorescence [8,12]. A plot of the rate constant against CETP concentration (inset Figure 4) indicates that the transfer rate is not linearly related to the amount of the transfer protein present. The flattening of the curve at high CETP concentrations suggests that the transfer is saturable with respect to CETP and probably reflects the maximum amount of CETP that can bind to the emulsion surface, an interpretation which is strengthened by the binding data presented in Figure 7. Evidence of saturation is also present in data reported previously [33,34].

We now direct attention to the effect of UC on the activity of CETP. The presence of UC enhanced the rate of transfer of Py-CE between emulsion particles. In these experiments, donor and acceptor particles contained the same UC:phospholipid ratio so that there was no net transfer of cholesterol with time. The presence of cholesterol also changed the kinetics of transfer such that a two-exponential function was required to fit the data (Figure 5; Table 1). The appearance of a fast early phase in the transfer kinetics when cholesterol is present is difficult to interpret at a microscopic level. The interpretation depends on the transfer model chosen. For example, it could be due to an increase in the off-rate constant for the dissociation of CETP from the donor if

CETP is considered to act as a shuttle between donor and acceptor. Regardless of the mechanism, it would appear that any increase in the microviscosity of the surface monolayer caused by cholesterol does not impede the transfer process. Cholesterol is also known to decrease the solubility of CE in bilayers and in the phospholipid monolayer of microemulsions [29,35,36]. Thus it is unlikely that CETP withdraws CE from the surface monolayer but obtains more direct access to CE located in the core compartment. Surface curvature effects might also play a part since UC increases the diameter of these emulsions by about 7% at a UC:phospholipid ratio of 0.15 [29].

An important question is whether the enhancement of transfer induced by cholesterol arises from increased binding of CETP to emulsion particles. The binding studies show that the presence of cholesterol reduces CETP binding. Using the values of K_d and the binding capacity (see the Results section) the amount of CETP bound under the conditions of the Py-CE assay was calculated to be 19% in the presence of 14 mol% cholesterol compared with 58 mol% in its absence. Clearly, the cholesterol effect cannot be attributed to improved binding to the emulsion. Apart from a specific interaction of cholesterol with CETP, cholesterol may affect transfer activity by altering the packing of phospholipids in the surface monolayer and by changing the surface curvature of the microemulsions. Cholesterol is known to decrease the cone angle of phospholipid motion in bilayers and to increase the radius of curvature of DMPC-CO microemulsions [29,30]. Apolipoproteins are also known to increase the activity of CETP [8,9,12] but it remains to be determined whether the cholesterol effect is still apparent under such conditions.

Preincubation equilibrates the levels of UC in donor and acceptor particles. The larger pool of cholesterol in the excess of HDL acceptor equilibrates with a relatively small pool in the donor emulsion bringing the cholesterol content of different emulsion preparations to similar levels (Figure 5b). Thus the rate of CE transfer was similar regardless of whether the initial contained 0 or 14 mol% cholesterol. The question of whether the cholesterol effect determines rates of CE exchange between lipoproteins, or whether the effect is masked by dominant effects of apolipoproteins remains to be determined.

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